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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
α6β4 integrin activates PI3-K, which facilitates breast carcinoma cell migration and invasion. In this report we characterize one more pathway acting downstream of α6β4/PI3-K. We show that RhoA activation is needed as an initial signal for induction of lamellae, but PI3-K dependent activation of PKCε is needed for stabilization of lamellae and hence for efficient carcinoma cell migration and invasion. PKCε is constitutively active in carcinoma cells, and provides a separate, parallel signal that acts independently of RhoA activity. RhoA activation is an absolute requirement for initial steps of lamellae formation, but efficient cell motility and invasion requires PKCε activity. Other PKC isoforms cannot replace PKCε, and therefore PKCε provides a promising target for pharmacological treatments and gene therapy of breast cancer.

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Introduction

Our research focuses on one of the key issues in breast carcinoma biology, the interaction of the breast carcinoma cell with its surroundings. Carcinoma cells actively produce extracellular matrix components, especially laminins, and use their integrin receptors for adhering to these newly deposited matrix proteins (Tani et al., 1999; Tani et al., 1997). Adhesion to laminins and other matrix molecules not only provides the cells with support and physically enables cell migration, but also provides signals that regulate carcinoma cell growth, differentiation, invasion through surrounding tissues and eventually metastasis. Our main focus, the integrin receptors, is important for breast carcinoma progression in at least two ways. Upon ligation by extracellular matrix molecules integrins activate specific signal transduction pathways, including PI3-kinase, p42/p44 MAP kinase and p38 MAP kinase (Ivaska et al., 1999; Shaw et al., 1997; Wei et al., 1998). On the other hand, integrins modulate the immediate vicinity of the cells, e.g. by concentrating laminin heterotrimers near the plasmamembrane thus facilitating laminin polymerisation and basement membrane formation.

During the two years with Taneli T. Tani as the PI of this project, two major projects on laminin receptor function were completed. The first project aimed at finding cytoplasmic proteins specifically binding to the type I PDZ recognition sequence at the C-terminus of α6A integrin subunit. Surprisingly, TIP-2/GIPC was found to interact with both α6 isoforms, and the α6B subunit was demonstrated to have a novel type of PDZ recognition sequence. The second project was carried out in collaboration with Dr. Leslie M. Shaw. Dr. Shaw had preliminary data on the role of PKCε in carcinoma cell motility and invasion. On the other hand, my own preliminary data pointed towards synergistic effects of RhoA and PKC activation in invading cells. Taken together, our data shows that PKCε provides a parallel signal regulated in part by PI3K that facilitates the RhoA driven migration and invasion of carcinoma cells.

Body

Because our previous data suggested that α6A and α6B cytoplasmic domains are likely to have specific signaling functions, we took this as our starting point in characterizing the interplay on laminin receptor function in breast carcinoma. As is true for other integrin α subunits, the cytoplasmic domains of α6A and α6B are short (36 and 56 amino acids, respectively), devoid of enzymatic activities and docking sites for cell signaling molecules. Surprisingly little is known about direct protein-protein interactions between integrin α-subunits and cytoplasmic proteins. However, we realized that there is a classical type I PDZ domain at the end of α6A integrin subunit (TSDA*), characterized by serine at –2 position and an aliphatic residue at the C-terminal 0 position. Yeast two-hybrid screen suggested that TIP-2/GIPC, a previously characterized cytoplasmic protein with a single PDZ domain, binds to the C-terminal sequences of α6A, α5 (TSDA*) and α6B (ESYS*) integrins. The recognition sequence in α6B (ESYS*) has not been previously characterized as a PDZ recognition sequence, and probably respresents a new subclass of type I PDZ binding site.

This project on the putative PDZ domains in integrin α subunits was finished during the period of May 15, 2001 to May 14, 2002. In addition to the data provided in the first annual report, a definitive proof of the PDZ interaction was provided by demonstrating that the PDZ domain of TIP-2/GIPC is sufficient for interaction with both α6A and α6B integrin subunits. In addition, the properties of the novel PDZ recognition sequence in α6B C-terminus were characterized by a more thorough point mutation analysis. The results were published in September 2001 (Tani and Mercurio, 2001).

Most of the work during the period of May15, 2001 to May 14, 2002 focused on the role PKCs in breast carcinoma cell invasion. Previous studies have shown that α6β4 integrin activates PI3-K, and eventually leads to facilitated carcinoma cell migration and invasion (Shaw et al., 1997). Several lines of evidence suggest a role for PKCs in the regulation of cell migration, and because novel and atypical PKCs are regulated by PI3-K (Cenni et al., 2002; Chou et al., 1998; Derman et al., 1997) we hypothesized that one or more of these kinases might act downstream of the α6β4/PI3-K pathway. Some of our own unpublished data also supported this hypothesis. Clone A colon carcinoma cells do not form lamellae on collagen I. However, a short treatment with PMA rapidly induces large lamellae and rapid cell migration even on collagen I. This phenomenon is accompanied by activation of RhoA. On the other hand, we had preliminary data that kinase inactive alleles of PKCε inhibit haptotactitc migration and invasion of MDA-MB-435 breast carcinoma cells, whereas kinase inactive isoforms of other PKCs fail to do this.

The preliminary biochemical experiments on RhoA and PKCs were done with CloneA cells, and therefore we started the study by characterizing the expression pattern of different PKC isoforms in CloneA cells. Novel PKCs δ and ε are expressed in CloneA cells, whereas η and θ are not. The atypical PKCζ, also regulated by PI3-K, is expressed in CloneA cells.

Although CloneA cells do not invade in Boyden chamber models of invasion, they do show haptotactic migration towards a laminin-1 gradient. We expressed several PKC constructs in Clone A cells and compared their ability for haptotactic mmigration. Kinase inactive PKCε reduced haptotactic migration by 80%, whereas the kinase inactive PKCδ and PKCζ constructs had no effect. This result suggests that PKCε has a unique role among PKCs in facilating the migration of carcinoma cells.

As the next step, we aimed at characterizing the activation of PKCε in different conditions. Activation of PKCs is a complex process. It starts with the maturation consisting of three strictly regulated phosphorylation steps, and subsequent recruitment to membrane through calcium or diacyl glycerol (DAG) dependent interactions. The final step in the activation of PKCε is the interaction with DAG, and therefore incorporation to the mebrane fraction in vivo can be used as a crude estimate of activity of this enzyme in vivo. In our experiments Cllone A cells were either kept in suspension or plated on collagen or laminin-1 for 30-45 minutes. PKCα was not found in the membrane fraction in any of the conditions, whereas a pool of PKCε was found associated with the membrane in all conditions tested. These results suggest that PKCε is partly active in a constitutive manner, and the interaction with the extracellular matrix does not affect it.

We also tested the effect of brief treatment LY294002, a potent and specific inhibitor of PI3-K activity. This short treatment did not change the amount of PKCε in membrane fraction. Although PI3-K activity is needed for the phosphorylation of PKCε by PDK-1 (Cenni et al., 2002), this is a very early step in the activation of PKCε, and therefore it is not surprising that short term inhibition of PI3-K does not cause any changes in the membrane association of PKCε.

Morphology and video microscopy of Clone A cells was performed by Dr. Leslie M Shaw. Kinase inactive PKCε caused a collapse of lamellae, and an increase in the number of retraction fibers suggestive of problems with detachment from the extracellular matrix. Kinase incative PKCζ did not cause any of these effects, which supports the hypothesis that PKCε has a specific role in cell migration. In cells transiently transfected with kinase inactive PKCε the lamellae initially form normally, but they are unstable and rapidly collapse. This collapse inhibits cell motility and prevents the cells from efficiently using their actin cytoskeleton for cell motility.

Previous studies from this group have suggested causal relationship between RhoA activation and induction of lamellae in Clone A cells (O'Connor and Mercurio, 2001; O'Connor et al., 2000). The relationship between PKCε and RhoA activation is therefore especially interesting. PMA has been known for a long time to activate RhoA (Nishiyama et al., 1994), but the exact pathway responsible for activation is not known. On the other hand, our data so far demostrates that a pool of PKCε is constitutively in its membrane bound, activated form. We studied the activation of RhoA upon adhesion to laminin-1 using the well-characterized Rhotekin pulldown assay. Our data suggest that RhoA activity is at its highest 30 minutes after plating on laminin-1, and slowly decreases after that as previously described. PKC inhibitors (Go6976 and Go 6983) do not affect RhoA activation induced by interaction with laminin-1. In cells plated on collagen for two hours the RhoA activity is extremely low. In these cells RhoA can be rapidly activated by treatment with PMA. Activation of RhoA by PMA is completely blocked by broad spectrum PKC inhibitor Go6983. We therefore conclude that activation of RhoA upon adhesion to laminin-1 is independent of PKC activity, and PKCε creates a separate, parallel signal that facilitates cell migration.

As the final step, we studied these signaling pathways in carcinoma cell invasion. MDA-MB-435 cells were used in Matrigek invasion assays as previously described. Kinase inactive PKCε reduced the invasion by 50%, whereas kinase inactive PKCζ had no effect.

Key research accomplishments

The results of the studies on PKCε in carcinoma cell migration and invasion can be summarized as follows:

1. PKCε is constitutively active in carcinoma cells. Because the early steps of PKCε activation are PI3-K dependent, the α6β4/PI3-K pathway facilitates PKCε signaling in the long run.

2. Although a pool of PKCε is in its active, membrane bound form in Clone A cells plated on collagen, these cells do not form lameallae because the initial signal for lamellae formation (RhoA activation) is missing
3. PMA treatment activates RhoA, which induces lamellae even when cells are plated on collagen.
4. PKCε and RhoA act on separate, parallel pathways in facilitating carcinoma cell migration and invasion.
5. RhoA is the initial signal for induction of lamellae, whereas PKCε stabilizes the lamellae and enables efficient use of actin cytoskeleton for cell motility.
6. Inhibition of PKCε activity reduces breast carcinoma cell invasion.

Reportable outcomes

Tani, T.T., and A.M. Mercurio. 2001. PDZ Interaction Sites in Integrin alpha Subunits. TIP-2/GIPC BINDS TO A TYPE I RECOGNITION SEQUENCE IN alpha 6A/alpha 5 AND A NOVEL SEQUENCE IN alpha 6B. *J Biol Chem.* 276:36535-36542.

Conclusions

Our previous studies have demonstrated the importance of α6β4 integrin for breast carcinoma invasion. In this project we have described one more pathway that operates downstream of α6β4/PI3-K pathway. The fact that PKCε has a specific role in breast carcinoma invasion provides a target for pharmacological and gene therapy in advanced breast cancer. Although PKC isoforms are highly similar in their structure, our work demonstrates that it is possible to find specific roles for each of them. PKCε is a promising target for future research on the regulation of actin cytoskeleton in invasive breast carcinoma cells.

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